ca. 10 cm long, and there was a constriction just above it to facilitate sealing; the total volume up to the constriction was 20 cm^3. Crystals of **2** (30 mg) were placed in the left-hand leg, and after 3 freeze–pump–thaw cycles the cell was sealed under vacuum. An electrical heating tape was wound around the horizontal section, the right-hand leg was immersed in liquid N_2, and the horizontal section was then heated to 450 °C. With the aid of a hot-air gun, some of the peroxide (6.1 mg) was passed through the hot zone.

Raman Spectra. Raman spectra were recorded by using Spex 1401 (1200 lines mm^{-1}) and 14018 (1800 lines mm^{-1}) spectrometers in conjunctions with a Coherent Radiation model CR12 argon ion laser. Detection of the scattered radiation was by photon counting, with RCA C31034 photomultiplier tubes.

Raman spectra of the gaseous photolysis products were obtained by focusing the laser in the center of the reaction tube, ca. 3 cm from the meniscus of the solution. The samples were aligned so that the laser beam did not pass through any of the solution. For a 90° scattering geometry with vertical slits and a single pass of ca. 700 mW of 514.5-nm radiation, it was found to be sufficient to clamp the reaction tube inclined ca. 15° from horizontal. The thermolysis sample was studied by focusing the laser in the center of the horizontal section of the reaction cell.

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Registry No. 1, 280-53-5; **1**-d_1, 95589-82-5; **1**-d_2, 95589-83-6; **2**, 283-35-2; H_2, 1333-74-0; HD, 13983-20-5; D_2, 7782-39-0; O_2, 7782-44-7; 1,4-dideuteriated-1,3-cyclohexadiene, 89829-68-5; 1,4-dideuteriated-1,4-cyclohexadiene, 17647-19-7; 1,4-dideuterio-2,3-dioxabicyclo[2.2.2]-oct-5-ene, 95589-84-7; cyclohexane-1,4-dione, 637-88-7.

Fusogenic Behavior of Didodecyldimethylammonium Bromide Bilayer Vesicles

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Abstract: The ability of surfactant vesicles, prepared from didodecyldimethylammonium bromide (DDAB), to undergo fusion in the presence of a variety of organic and inorganic anions has been investigated. The occurrence of fusion was examined by electron microscopy. The kinetics of fusion was monitored continuously by following the decrease of energy transfer between two fluorescent phospholipid analogues occurring when DDAB vesicles, containing these fluorophores, fuse with nonlabeled vesicles. Vesicle fusion was induced upon addition of the divalent anion of dipicolinic acid (DPA), resulting in the formation of "giant" vesicles with a diameter of 2–3 μm. The DPA dianion (DPA^{2-}) displayed a threshold concentration of ca. 30 μM, below which fusion could not be detected. Since vesicle aggregation, as monitored by absorbance measurements, revealed a threshold DPA^{2-} concentration of ca. 15 μM, it is likely that fusion represents the rate-limiting step in the overall process. Hence, after vesicle aggregation, structural membrane alterations must occur which subsequently render the membranes susceptible to fusion. Remarkably, only vesicles with a diameter of at least 3000 Å are prone to fusion, whereas smaller vesicles, despite the fact that they readily aggregate, do not fuse, even at high DPA^{2-} concentrations. Preliminary results revealed that anions other than DPA^{2-} were also capable of inducing fusion of DDAB vesicles, provided that they display dual functional properties by containing either a divalent charge or a monovalent charge in addition to a group which presumably prefers hydrophobic membrane interactions.

An interesting area of membrane mimetic chemistry has emerged from the development of simple membrane-forming amphiphiles.[1] Surfactant vesicles, formed upon sonic dispersal of a variety of single- and double-chained synthetic amphiphiles, have been shown to resemble in many respects membrane bilayers formed from synthetic and natural phospholipids. For example, amphiphilic bilayers undergo thermotropic phase transitions[2] while, depending on their size, they also display osmotic sensitivity.[2a,3] Moreover, it has been demonstrated that closed vesicle bilayers can be formed from a double-chained amphiphile carrying an *N*-methylpyridinium head group and that bilayers from this surfactant could be reconstituted containing the biologically active protein rhodopsin. These results indicate that these surfactant vesicles may be employed to mimic cell-membrane functions.[4]

Studies with synthetic membrane mimics may provide relevant information concerning the properties of lipid/water interfaces and structural effects on membrane stability. With respect to the latter, it is relevant to note that surfactant vesicles are generally much more stable than phospholipid vesicles. Biological membranes can display an inherent but transient destabilization, occurring during membrane fusion which takes place during physiologically important processes such as endocytosis, exocytosis, and virus infection.[5] Although phospholipid vesicles have been used extensively as models in studies of membrane fusion mechanisms,[6] some recent investigations have suggested that bilayers prepared from synthetic amphiphiles may also become transiently instable and, subsequently, fuse.[7]

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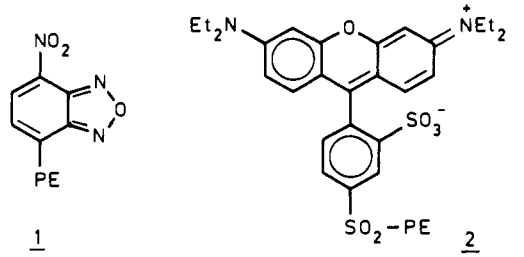
In the present work, we have examined the potential fusogenic properties of didodecyldimethylammonium bromide (DDAB) vesicles as induced by organic and inorganic anions. To unequivocally establish fusion, we have employed an assay based on resonance energy transfer.[8] This procedure allows continuous monitoring of the decrease in energy-transfer efficiency between two nonexchangeable fluorescent phospholipid analogues *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (**1**, *N*-NBD-PE; donor) and *N*-(Lissamine Rhodamine B sulfonyl)-phosphatidylethanolamine (**2**, *N*-Rh-PE; acceptor) when a labeled



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membrane fuses with a nonlabeled membrane. The results indicate that fusion susceptibility is conferred to the vesicles within the aggregated state and that the propensity to undergo fusion is strongly dependent on the size of the vesicles.

Experimental Section

Materials. Didodecyldimethylammonium bromide (DDAB) was purchased from Eastman Kodak and recrystallized twice (acetone) before use. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (**1**, *N*-NBD-PE) and *N*-(Lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (**2**, *N*-Rh-PE) were obtained from Avanti Polar Lipids Inc. Dipicolinic acid (DPA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were from Sigma Chemicals. Picolinic acid was obtained from Fluka, sodium sulfate from Baker, and butyric acid, sodium phosphate, and benzoic acid from Merck. All chemicals were of the highest grade available and were used as such.

Vesicle Preparation. Two DDAB vesicle preparations of different size were obtained by rapid injection of an ethanolic solution of the amphiphile into twice-distilled water.[9] Either 10 or 30 mg of DDAB was dissolved in 75 μL of ethanol (final concentrations 0.29 and 0.87 M, respectively). Fifty microliters were injected into 2 mL of water under stirring. As determined by electron microscopy, the majority (ca. 70%) of the vesicles thus formed had an average diameter of ca. 2200 Å and ca. 3200 Å, upon injection of 0.29 and 0.87 M DDAB, respectively. Since exhaustive dialysis of the vesicle suspensions against aqua-bidest in order to remove the ethanol did not alter the experimental results, this procedure was omitted in most of the experiments, and the vesicles were diluted to the appropriate concentrations immediately after preparation.

Vesicle Aggregation. Aggregation of DDAB vesicles, induced by various anions in Hepes buffered solutions, pH 6.0, at 25.5 °C, was measured by monitoring continuously the turbidity changes at 400 nm by using a Perkin-Elmer Lambda 5 spectrophotometer, equipped with a thermostated cell holder. The absorbance change obtained 2 min after induction of vesicle aggregation by addition of the vesicle solution to a solution of the appropriate anion was taken as a measure of the initial rate of aggregation.

Fusion Measurements. Vesicle fusion was monitored by using the resonance energy-transfer assay described by Struck et al.[8a] This method takes advantage of the overlap between the emission and excitation spectrum of two, nonexchangeable fluorescent phospholipid analogues, *N*-NBD-PE, (**1**; donor) and *N*-Rh-PE, (**2**; acceptor). The fluorophores can be incorporated into membranes at concentrations that do not perturb the bilayer structure, while the efficiency of energy transfer is proportionally related to their surface density. Upon fusion of fluorescently labeled DDAB vesicles with nonlabeled vesicles, bilayer mixing will reduce the surface density of the fluorophores, resulting in a decrease of resonance energy-transfer efficiency. The latter can be followed continuously as an increase in NBD fluorescence, thus allowing accurate monitoring of the fusion process.

Vesicles containing 0.8 mol % each of *N*-NBD-PE and *N*-Rh-PE were prepared by solubilizing appropriate amounts of DDAB and fluorophores in ethanol. Fusion measurements were carried out in Hepes buffered solutions (pH 6.0, 25.5 °C) with a 1:1 mixture of labeled and nonlabeled DDAB vesicles in aqua-bidest at a final amphiphile concentration of 50 μM. Fusion was initiated by injecting the vesicle solution into the solution of the appropriate (di)anion by using a Hamilton syringe. NBD fluorescence (λ_{ex} = 475 nm, λ_{em} = 530 nm) was monitored continuously by using a Perkin-Elmer MPF43 spectrofluorometer with a thermostatically controlled cell holder equipped with a magnetic stirring device. The fluorescence scale was calibrated such that the residual NBD fluorescence of the vesicles is taken as the zero level and the value after addition of Triton X-100 (final concentration 1% v/v), corrected for the sample dilution and the effect of Triton X-100 on the NBD fluorescence, as 100% (infinite dilution). At a ratio of labeled vesicles to nonlabeled vesicles of 1:1, total mixing of the amphiphilic membranes would result in the fluorescence reaching 50% of its value at infinite probe dilution and hence corresponds to 100% fusion.

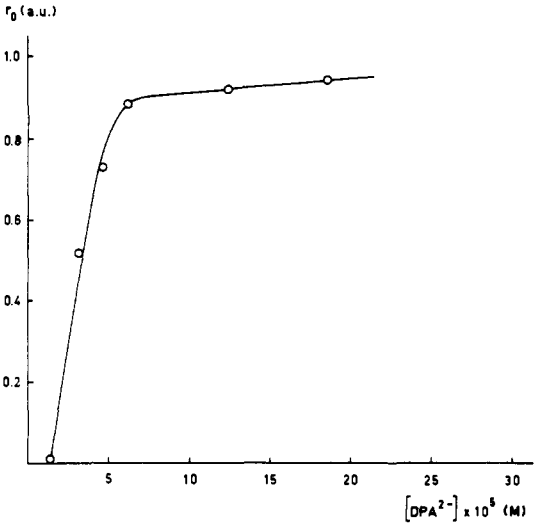

Figure 1. Effect of the DPA^{2-} concentration on the initial rate of aggregation (in arbitrary units) of large DDAB vesicles. [DDAB] = 55 × 10^{-6} M, pH 6.0, incubation temperature 25.5 °C.

Electron Microscopy. Aliquots of DDAB vesicles, before and after addition of anions, were negatively stained with a 1% (by weight) solution of uranyl acetate, as previously described.[4a] The samples were examined in a Philips EM 300 electron microscope operating at 80 kV.

Results and Discussion

Vesicle aggregation was not observed when small (diameter ca. 2200 Å) or large (3200 Å) DDAB vesicles, prepared by ethanol injection, were stored for several days at room temperature. However, in the presence of relatively small amounts of the almost fully (95%) ionized (pH 6.0) dipicolinic acid (DPA), both the smaller and the larger vesicles (Figure 1) aggregate rapidly as indicated by an increase in turbidity. The kinetics of aggregation were markedly dependent on the DPA^{2-} concentration, exhibiting a threshold concentration of ca. 13 μM below which aggregation was not observed. The initial rate of aggregation increased sharply in the range of 13–50 μM DPA^{2-}, until a maximum rate is reached beyond 50 μM.

Examination of the aggregates by phase contrast microscopy revealed the presence of grainlike aggregates, formed from the smaller vesicles in the presence of DPA^{2-}. In contrast, much larger vesicle structures of several *micrometers* in diameter were seen upon treatment of the larger DDAB vesicles with the dianion, indicating that DPA^{2-}-induced *fusion* of these vesicles had taken place. As shown in Figure 2, electron-microscopic examination of the preparations confirmed these observations. Vesicles of smaller sizes were generally seen to be adhered to the large fused structures, conceivably originating from contamination of the original vesicle population with small vesicles. Their mere adhesion is consistent with the resistance of smaller vesicles to fusion. Studies aimed at elucidating the mechanism of fusion of phospholipid membranes have emphasized the importance of investigating initial fusion events in order to correlate early changes in the structural and physical properties of the membrane bilayer lipids with the onset of membrane fusion.[8b,10] Therefore, we

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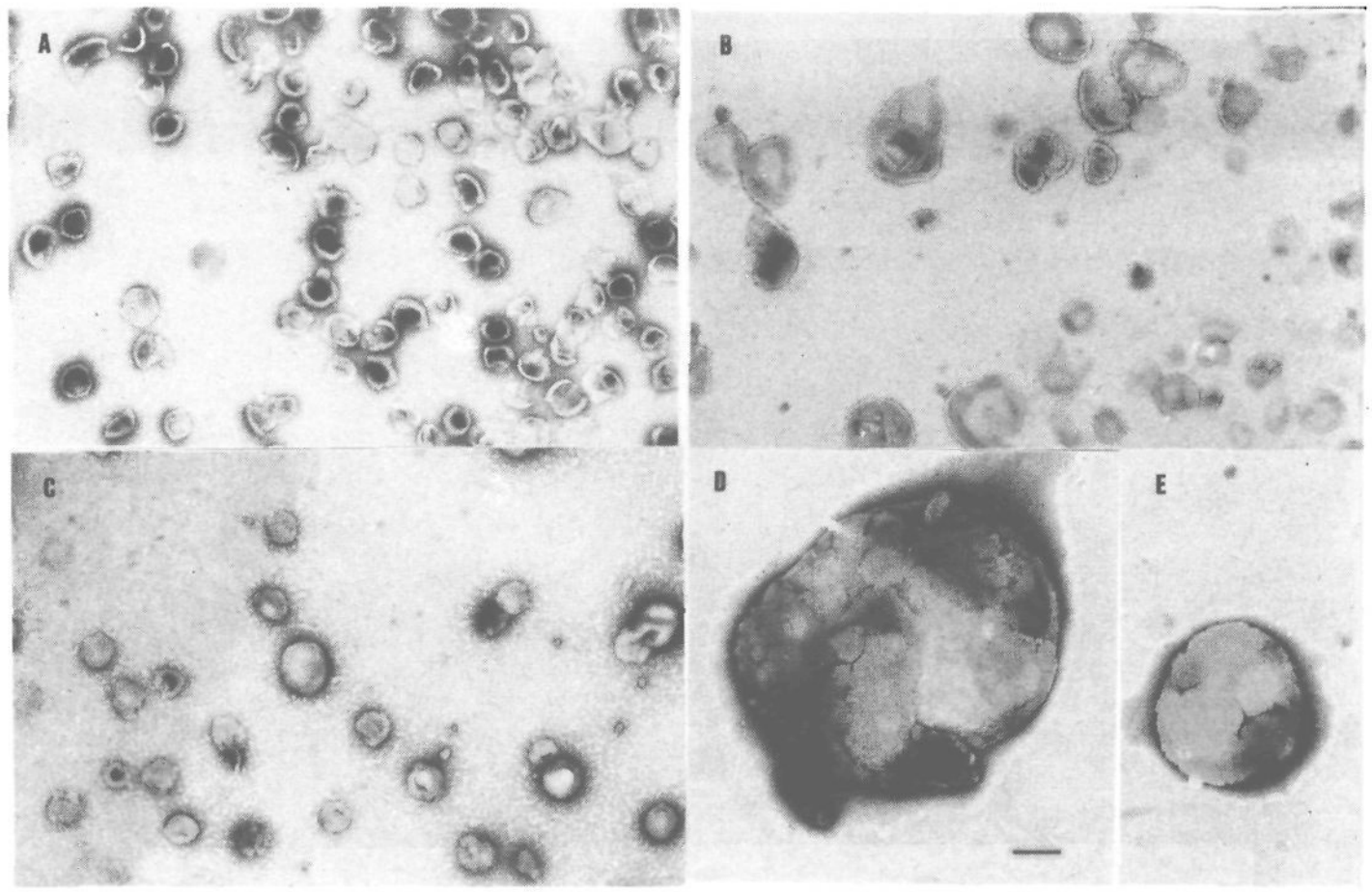

Figure 2. Electron micrographs of DDAB vesicles prepared by injection of (A) 0.29 M DDAB (in ethanol) and (B) 0.87 M DDAB (in ethanol) in aqua-bidest. (C), as in (A), after incubation with DPA^{2-}; (D) and (E), as in (B), after incubation with DPA^{2-}. In all cases the initial magnification is 28.800×. The marker line represents 3500 Å.

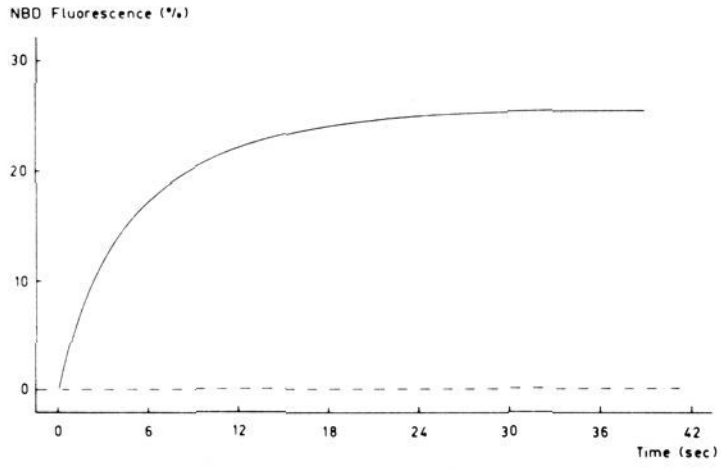

Figure 3. Time-dependent *N*-NBD-PE fluorescence change after addition of DPA^{2-} to a 1:1 mixture of labeled and nonlabeled DDAB vesicles. Total [DDAB] = 50 μM; [DPA^{2-}] = 6.25×10^{-5} M, pH 6.0. Solid curve: [DDAB] in ethanol is 0.87 M; dashed curve: [DDAB] in ethanol is 0.29 M.

performed a kinetic analysis of the DPA^{2-}–induced fusion of DDAB vesicles by using a membrane fusion assay based on resonance energy transfer between the fluorophores *N*-NBD-PE, (**1**, donor) and *N*-Rh-PE (**2**, acceptor). When either the smaller or larger DDAB vesicles, containing both *N*-NBD-PE and *N*-Rh-PE, incubated with an equal amount of nonlabeled vesicles, no increase in NBD fluorescence could be detected. This indicates that the probes did not spontaneously transfer via a mechanism involving diffusion of monomers through the aqueous phase between labeled and nonlabeled membranes. Neither could dilution of the fluorophores be detected upon addition of DPA^{2-} to the smaller vesicle population (Figure 3), despite the fact that extensive vesicle aggregation was observed under these conditions (vide supra). These observations exclude the possibility of transfer of fluorophore via a collision-mediated mechanism and, moreover, confirm the inability of smaller DDAB vesicles to fuse in the presence of DPA^{2-}. By contrast, addition of DPA^{2-} to the fraction of larger vesicles resulted in rapid increase in NBD fluorescence (Figure 3), indicating the occurrence of fusion between labeled

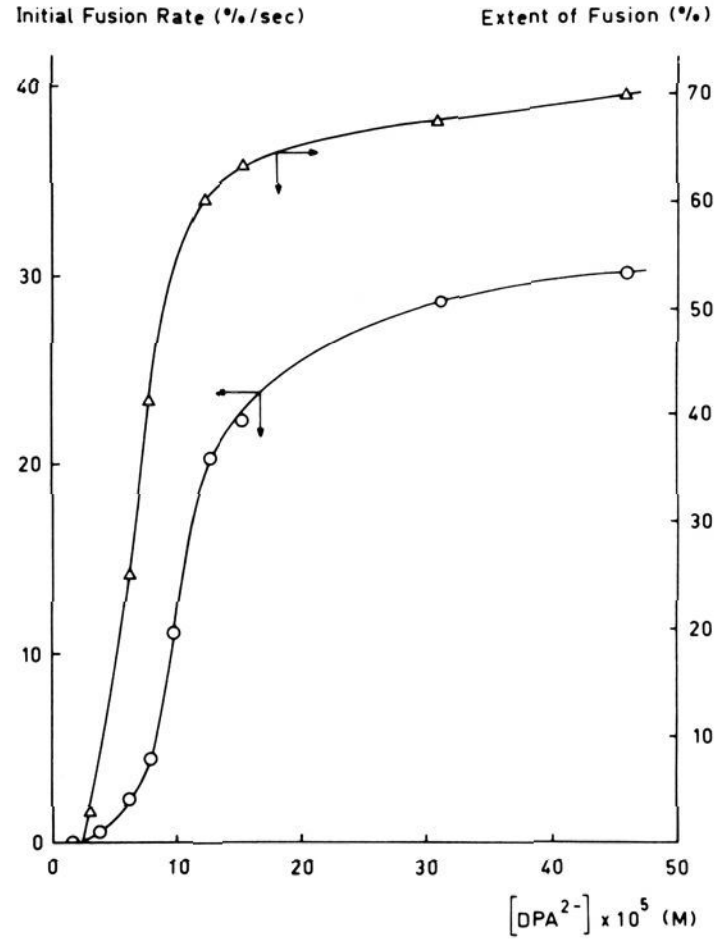

Figure 4. Effect of the DPA^{2-} concentration on the initial fusion rate (○) and the extent of fusion (△) of DDAB vesicles. The initial rate of fusion was calculated from the tangents drawn to the curves as shown in Figure 3 at $t = 0$. The extent of fusion was calculated from the maximal level of fluorescence reached in the presence of DPA^{2-}, relative to the fluorescence obtained at infinite dilution, as determined after addition of Triton X-100. The ratio of labeled to nonlabeled vesicles was 1:1; total [DDAB] = 50 μM, pH 6.0, incubation temperature 25.5 °C.

and nonlabeled vesicles, which is consistent with the electron-microscopic observations described above. The initial rate and the final extent of fusion, as a function of the DPA^{2-} concentration, are shown in Figure 4. These results demonstrate that ca. 70% of the DDAB vesicles are prone to fusion. Since it is reasonable to assume that the labeling efficiency of the small and large vesicles is similar,[8a] we suggest that the 30% fraction of the vesicles not

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participating in fusion corresponds to the fraction of smaller vesicles, present as contaminant in the initial vesicle population.

The overall process which eventually leads to membrane fusion involves two consecutive steps: membrane aggregation and merging of the bilayers. Comparison of the initial rates of fusion with those of aggregation, both as a function of the DPA^{2-} concentration, leads to the following conclusions. Firstly, the threshold DPA^{2-} concentration for fusion (ca. 30 μM, Figure 4) is significantly higher than that for aggregation (ca. 13 μM). Secondly, the rate of aggregation reaches a maximum value around 50 μM DPA^{2-}, whereas the rate of fusion increases strongly in the range of 70–130 μM DPA. These results indicate that the fusion event represents the rate-limiting step in the overall process. Thus, fusion susceptibility is conferred to the vesicles within the aggregated state, just as in the case of fusion of phospholipid vesicles.[6b]

While charge neutralization of negatively charged phospholipid membranes or positively charged DDAB vesicles will suffice to induce membrane aggregation, this process is not necessarily accompanied by membrane fusion, as indicated by the absence of fusion of (1) phospholipid vesicles in the presence of high salt concentrations[11] and (2) the smaller DDAB vesicles in the presence of DPA^{2-}. Obviously, a second barrier has to be overcome before merging of apposed bilayers will occur. In phospholipid vesicle systems, this barrier is thought to involve strong hydration forces, which lead to repulsion between hydrated phospholipid head groups in apposed bilayers.[6b,12] In those systems, dehydration can be accomplished by the formation of specific cation–phospholipid complexes, which, in turn, leads to local fluctuations in lipid packing at the site of interbilayer contact. At present, it is not clear whether similar mechanistic features apply to DPA^{2-}–induced fusion of DDAB vesicles. It is obvious, however, that DPA^{2-}–facilitated structural fluctuations in DDAB bilayers have to take place to initiate the fusion process. Apparently, this particular property is not exclusively restricted to DDAB–DPA^{2-} complex formation, as revealed by the fusogenic capacity of structurally related and nonrelated compounds. The anions of benzoic acid and *p*-toluenesulfonic acid as well as Na_2SO_4 induce both aggregation and fusion at rates which were similar to those obtained for DPA^{2-} at comparable concentrations. The (mono)anions of picolinic acid and propionic acid as well as Na_3PO_4 (monoanion at pH 6) appeared to be inactive, although some fusion was seen (ca. 5%) at a relatively high picolinic acid concentration of 200 μM. Thusfar the results indicate that those compounds capable of inducing fusion contained either a divalent charge (DPA^{2-} and Na_2SO_4) or a monovalent charge in addition to a side group with hydrophobic character (benzoic acid, *p*-toluenesulfonic acid). These dual functional properties would allow to bring two adjacent bilayers into close proximity either by electrostatic interactions or by bridging via a combination of electrostatic and hydrophobic interactions. It remains to be established whether subsequent events, leading to fusion, involve physical changes such as those occurring during fusion of phospholipid vesicles.

Concluding Remarks

The most interesting observation reported in this communication is that cationic surfactant vesicles can be fused upon addition of appropriate anions. It is remarkable that fusion of DDAB vesicles, with an average size of ca. 3200 Å, results in the formation of "giant" vesicles (2–3 μm) whereas the fusion products of phospholipid vesicles are generally an order of magnitude smaller. Presumably, this difference arises from the much higher inherent stability of the synthetic surfactant bilayers. The observation that fusion of DDAB vesicles is restricted to vesicles with a diameter of at least 3200 Å is rather intriguing since the fusion susceptibility of phospholipid vesicles tends to *decrease* rather than to increase with increasing vesicle size.[10a] These results suggest differences in bilayer constraints between phospholipid and synthetic surfactant vesicles, which are presumably due to differences in head group interactions. Moreover, they also indicate that such differences may exist between the smaller and larger DDAB vesicles. The resistance of the smaller vesicles to undergo fusion, emphasizing their inherent stability, is consistent with observations published by Murakami et al.[13] However, fusion of small surfactant vesicles has been reported by Shimomura and Kunitake,[7a] but the conditions were not comparable to those employed in the present study. Unfortunately, these authors did not rigorously exclude the possibility of transfer of free monomers.

For larger vesicles, it is reasonable to assume that the smaller curvature results in a higher packing density of the amphiphilic molecules. To minimize the electrostatic repulsions between the head groups, a lower degree of head group ionization would therefore be expected.[14] As a result, differences in the molecular packing mode may lead to comparatively loose molecular arrangements which may weaken hydrophobic interactions between the amphiphilic molecules. Upon addition of a membrane-aggregating agent, close approach of adjacent bilayers may facilitate further structural rearrangements which can drastically modify the interactions between apposed membranes.[12] Whether such defects, required to induce fusion, involve the formation of specific complexes remains to be established. However, preliminary studies of the interaction of DPA^{2-} with small and large DDAB vesicles have revealed that the phase-transition temperature is shifted toward a higher temperature upon binding of DPA^{2-} to the large vesicles but *not* to the smaller ones. The relevance of this observation with respect to the mechanism of anion-induced fusion of synthetic surfactant vesicles is currently under investigation.

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